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Incorporation of Sulfide Ions into the Cadmium(II)-Thiolate Cluster of *Cicer arietinum* Metallothionein2

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This work is dedicated with the best wishes to Professor Helmut Sigel on the occasion of his 75th birthday

ABSTRACT

The plant metallothionein2 from *Cicer arietinum* (chickpea), cic-MT2, is known to coordinate five divalent metal ions such as Zn^{II} or Cd^{II}, which are arranged in a single metal-thiolate cluster. When the Zn^{II}-form of the protein is titrated with Cd^{II} ions in presence of sulfide ions, an increased Cd^{II} binding capacity and concomitant incorporation of sulfide ions into the cluster is observed. The exact stoichiometry of this novel cluster, its spectroscopic properties and the significantly increased pH stability is analysed with different techniques, including UV and CD spectroscopy and calorimetric assays. Limited proteolytic digestion provides information about the spacial arrangement of the cluster within the protein. Increasing the Cd^{II} scavenging properties of an MT by additionally recruiting sulfide ions might be an economic and very efficient detoxification strategy for plants.

INTRODUCTION

Exposure to heavy metal ions such as Cd^{2+} induces the enzymatic synthesis of a class of small peptides known as phytochelatins (PCs), oligomers of glutathione with typically 2-6 repeating units and the general formula $(\gamma\text{-EC})_nG$. PCs have been identified particularly in higher plants, marine and freshwater algae, fission yeast, and filamentous fungi.[1] More recently it was found that the genome of the nematode *Caenorhabditis elegans* encodes a protein with PC synthase activity, conferring an increased Cd^{II} tolerance to the animal as well as to yeast cells in complementation assays.[2, 3]

The Cd-γ-glutamyl peptide complexes of fission yeast, *Schizosaccharomyces pombe*, were the first, in which an additional incorporation of sulfide ions was observed.[4] Two different complexes can be isolated from *S. pombe*, a high molecular weight complex containing sulfide ions, $Cd_{5.4}S[(\gamma\text{-EC})_3G]_4$, next to a smaller amount of a sulfide-free low molecular mass complex, $Cd_{1.8}[(\gamma\text{-EC})_3G]_2$.[4, 5] The high molecular mass complex can be transformed into the low molecular mass complex by acidification and re-neutralization, which removes the labile sulfide ions in form of hydrogen sulfide. *In vitro* treatment of the low molecular mass complex with Na_2S and Cd^{II} results in a complex with even higher Cd^{II} and sulfide binding abilities and a Cd^{II}:S^{2-}:$(\gamma\text{-EC})_3G$ ratio of 1.8:1:1.[4] Incorporation of sulfide ions into Cd^{II}-thiolate clusters causes a red-shift of the cluster-specific ligand-to-metal charge transfer (LMCT) bands observed in the UV spectra from 250 nm in the sulfide-free complexes to ~265 nm or even ~310 nm for the sulfide containing complexes found *in vivo* and prepared *in vitro* as described above, respectively. It was also shown that increasing $CdCl_2$ concentrations in the growth media (up to 2 mM) lead to a linear increase of the cellular sulfide production and shift the ratio of the two *in vivo* produced PC complexes towards the high molecular mass complex.[6] Hence, the incorporation of sulfide ions can be directly linked to increased Cd^{II} binding capacity of PCs in yeast, probably enhancing the effectiveness of the detoxification system. In addition, sulfide ions were shown to increase the thermodynamic as well as the pH stability of the Cd-γ-glutamyl peptide complexes.[7] A more detailed analysis of the *in vitro* complex with the Cd^{II}:S^{2-} ratio of 1.8:1 revealed the formation of nearly monodisperse particles with a diameter of ~18 Å.[8] They contain a crystallite CdS core and show an X-ray diffraction pattern closely similar to the six coordinate rock-salt structure. This is remarkable considering that naturally occurring CdS adopts a four coordinate zinc-blende structure and only converts to the rock-salt structure at high pressure.

Similar to *S. pombe* also plants produce low and high molecular weight Cd-PC complexes depending on the respective growth conditions. Sulfide ions were analogously identified mainly in

the higher molecular weight complexes, i.e. in *Lycopersicon esculentum* (tomato), *Silene cucubalus* (bladder campion), *Brassica juncea* (indian mustard), and *Rauvolfia serpentina* (snakeroot).[9-13] In the latter even three different kinds of complexes varying in the Cys-thiolate:S^{2-}:Cd ratios were identified: a low molecular weight complex with a ratio of 3:0:1, a medium molecular weight complex with 2.5:0:1, and a higher molecular weight complex with a ratio of 1.5:0.5:1. Hence with increasing molecular weight and most importantly upon incorporation of sulfide ions, the total S:Cd^{II} ratio is lowered. This let to the hypothesis that recruiting sulfide ions as additional ligands makes Cd^{II} detoxification far more economic for the organism than deploying the amino acid Cys as sole ligand.[13] Additionally, as sulfide ions are formed prior to Cys in the biosynthetic assimilatory sulfate reduction pathway substituting at least a part of the Cys ligands by sulfide ions decreases the energy consumption for the synthesis of metal chelators even further.

More recently, sulfide ions were also detected in metallothioneins (MTs) from different species, when the proteins were expressed recombinantly in *Escherichia coli*.[14] In contrast to PCs, MTs are a superfamily of ubiquitous genetically encoded Cys-rich proteins with a molar mass of usually less than 10 kDa. The range of proposed functions include among others the homeostasis of essential metal ions such as Zn^{2+} and Cu^{+}, detoxification of heavy metal ions as for example Cd^{2+}, Hg^{2+}, and Pb^{2+}, and scavenging of reactive oxygen species under stress conditions.[15, 16] Metal ions are coordinated in form of metal-thiolate clusters, more recently also His coordination of divalent metal ions was observed in certain MT forms.[17, 18] Plant MTs are characterized by a high sequence diversity and accordingly are further divided into four subfamilies, designated as p1, p2, p3, and pec or p4.[16, 19, 20] Usually, representatives of all four subfamilies can be detected in a single plant showing differential expression depending among others on the tissue localisation, growth stage, and growth condition. Peculiar is the occurrence of long Cys-free amino acid stretches or linker between Cys-rich regions.[21]

As observed so far and in contrast to the PC complexes, the effect of sulfide ions on the metal ion binding capacities of MTs is distinctively smaller. For example, mammalian MTs are able to coordinate seven Zn^{2+} or Cd^{2+} ions. In the presence of sulfide ions this binding capacity is retained, i.e. a range of 6.4 to 7.3 bound divalent metal ions was observed, while between 1.5 and 3.5 equivalents of sulfide ions are associated with the proteins.[22] When analyzed separately, however, the two domains of mouse MT4 can bind slightly more metal ions, i.e. coordination of 3.9 Cd^{2+} ions and 5 sulfide ions in the β-domain and 4.6 Cd^{2+} ions and 1.7 sulfide ions in the α-domain compared to three and four divalent metal ions in the absence of sulfide ions, respectively.[22] Results for the yeast MT CRS5 paint a similar picture. Conveying the divalent metal ion-to-Cys stoichiometry

from the metal-thiolate clusters of mammalian MTs to the 19 Cys residues of CRS5 a range of 6.3 to 6.9 metal ions is calculated, hence this protein should in theory be able to coordinate 6-7, Zn^{2+} or Cd^{2+} ions. Recombinant expression in non Zn^{II}-supplemented media yields lower amounts, i.e. observation of a Zn_4CRS5 species,[23] while Zn^{2+} and Cd^{2+} supplementation produces species with the stoichiometric composition $Zn_{5.7}S_{0.4}$ and $Cd_{7.4}S_{1.8}$, respectively.[24] The signals in the ESI-MS spectra with the highest intensity can be assigned to Zn_6- and Zn_7CRS5 for the Zn^{II}-form and Cd_7CRS5 for the Cd^{II}-form, respectively, while additionally lower intensity signals of Zn_6S_2CRS5 or Cd_7S_2- and Cd_8S_2CRS5 can be observed. More heterogeneous are the results obtained for the recombinant expression of the plant MT2 from *Quercus suber* (cork oak). This MT subfamily features 14 Cys residues and should be able to coordinate around 5 divalent metal ions according to the range of 4.7-5.1 metal ions calculated as for CRS5 above. However, significantly lower amounts of metal ions were detected in the Zn^{II}-forms, while among others a Cd_5MT2 species was observed in the ESI-MS spectra (Table 1).[25-28] In the presence of sulfide ions, binding of 2-3 sulfide ions enables the additional coordination of 1-2 Cd^{II} ions by the protein and hence increased the Cd^{II} binding capacity of this plant MT2 form by up to 40%. It should be noted that all sulfide containing MT forms described above were overexpressed in form of glutathione S-transferase (GST) tagged proteins in *E. coli*.

Table 1. Stoichiometric composition of Zn^{II} and Cd^{II} species obtained by recombinant protein expression of cork oak MT2 in *E. coli* as well as different species observed in ESI-MS spectra of the proteins.

stoichiometric composition[a]	ESI-MS (species with higher intensity signals)	ESI-MS (species with lower intensity signals)
$Zn_{3.5}$ [28]	Zn_4	Zn_3, Zn_5
$Zn_{4.2}$ [25]	Zn_4	
$Zn_{3.5}S_1$ [26, 27]	Zn_4	Zn_3, Zn_4S_2
$Cd_{6.3}$ [28]	Cd_6	Cd_7
$Cd_{5.6}S_3$ [27]	Cd_6S_4	Cd_5
$Cd_{5.3}S_{2.2}$ [26]	Cd_5	Cd_6S_4
$Cd_{6.3}S_{2.4}$ [26]	Cd_6S_4	Cd_7S_4
$Cd_{6.7}S_{2.9}$ [26]	Cd_6S_4	Cd_7S_4

[a] Metal ion concentrations were determined with inductively-coupled plasma atomic emission spectroscopy (ICP-AES) as well as the total sulfur content, i.e. Cys and Met residues, of the

acidified samples, which was used to calculate the protein concentrations. Sulfide ions were quantified as H_2S using gas chromatography coupled to a flame photometric detector (GC-FPD).

Based on the property of cork oak MT2 to coordinate higher amounts of Cd^{II} in presence of sulfide ions, we investigated this property further with the aim to characterise the formed Cd^{II}-sulfide-thiolate complex in more detail. We chose the MT2 protein from *Cicer arietinum* (chickpea), cicMT2, which was previously characterised in the absence of sulfide ions.[29] cicMT2 is able to coordinate five Zn^{II} or Cd^{II} ions in a cluster arrangement connecting the N- and C-terminal Cys-rich regions with each other.

EXPERIMENTAL SECTION

Chemicals and enzymes. LB Broth (Miller), 1,4-dithio-DL-threitol (DTT), and isopropyl-β-D-thiogalactopyranosid (IPTG) were purchased from Chemie Brunschwig AG (Basel, Switzerland), Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) from Calbiochem (VWR International AG, Lucerne, Switzerland), and Chelex® 100 resin from Bio-Rad (Reinach, Switzerland). All others chemicals were ACS grade from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). *Tritirachium album* proteinase K was obtained from Qbiogene (Lucerna Chem AG, Lucerne, Switzerland). The deionized water used for the preparation of all solutions was vacuum degassed for approximately 30 min and nitrogen saturated for at least 1 h. Where strictly anaerobic conditions were required, solutions were rendered oxygen free by three freeze-thaw cycles under vacuum.

Over-expression and purification of Zn_5-cicMT2. Construction of the plasmid containing the *cicmt2* gene was described previously as well as the growth condition for the over-expression of Zn_5-cicMT2 in *Escherichia coli* Bl21(DE3) cells, the purification, the determination of protein concentrations via thiol group quantification, and analysis of the metal ion content with flame atomic absorption spectroscopy (F-AAS) using an AA240FS spectrometer (Varian AG, Zug, Switzerland).[29]

Cd^{II} titration of Zn_5-cicMT2 in presence of S^{2-} ions. To a 10 μM solution of Zn_5-cicMT2 containing 1 mM Tris-HCl (pH 7.5), 10 equiv. of S^{2-} were added in form of a 40.7 mM Na_2S solution, which was standardized by iodine titration using $NaIO_3$.[30] After 10 min incubation at room temperature, up to 15 equivalents of $CdCl_2$ were added. After addition of each equivalent, the solution was incubated for 30 min at room temperature before a UV spectrum was recorded.

Preparation of the Cd/S-cicMT2 complex. The Cd/S-cicMT2 complex was prepared as described

above starting from 2 mL of a 30 µM Zn_5-cicMT2 solution. Subsequently, the sample was purified with size-exclusion chromatography (SEC) as described previously for Zn_5-cicMT2.[29] The protein purity and the molecular weight of Cd/S-cicMT2 were examined by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris-tricine buffer system.[31] Modification of samples to break up the cluster structure was carried out with 3-(bromomethyl)-2,5,6-trimethyl-1H,7H-pyrazolo(1,2-α) pyrazole-1,7-dione (monobromobimane, mBBr) without addition of EDTA or a reducing agent.[32] The stoichiometry of Cd/S-cicMT2 was determined as described below.

UV-vis and circular dichroism (CD) spectroscopic measurements. UV-vis absorption spectra were recorded on a Cary 500 scan spectrophotometer (Agilent Technologies AG, Basel, Switzerland) over the range of 200-400 nm at room temperature at a scanning speed of 600 nm min^{-1} and expressed as molar absorptivity (M^{-1} cm^{-1}). CD spectra were measured on a J-810 spectropolarimeter (Jasco Inc., Japan) over the range of 200-400 nm at room temperature at a scanning speed of 10 nm min^{-1} with four spectra accumulations and expressed as molar ellipticity (deg cm^2 $dmol^{-1}$).

Quantification of S^{2-} ions in the Cd/S-cicMT2 complex. (a) *2-PDS assay*: 500 µL of a SEC-purified Cd/S-cicMT2 sample were acidified with HCl to pH 1 in order to release the coordinated S^{2-} ions in form of H_2S. The acidified solution was then centrifuged under vacuum at room temperature for 20 min using the Eppendorf Concentrator 5301 to remove released H_2S. To estimate the volume decrease due to additional evaporation of water, the sample weight as well as the Cd^{II} concentration (F-AAS) was determined before and after the centrifugation step and used to correct the values obtained below. Thiol groups and S^{2-} ions were quantified with 2,2'-dithiopyridine (2-PDS).[29, 33] The 2-PDS assay was performed before and after acidification and centrifugation under vacuum to determine the combined content of Cys thiol groups and sulfide ions or solely the amount of Cys thiol groups, respectively. The difference of both measurements was used to calculate the amount of S^{2-} ions per protein in the Cd/S-complex. Amino acid analysis (AAA) was used to verify the protein concentration in the Cd/S-complex before S^{2-} ion release. (b) *Methylene blue assay*: S^{2-} ions were quantified as described with small modifications.[34] All reagents required for the assay as well as the samples were prepared inside a N_2-purged glove box. 0.1 mL of a 20 mM *N, N*-dimethyl-*p*-phenylene-diamine sulfate (DPD) solution in 7.2 M HCl was mixed with 1 mL of the Cd/S-cicMT2 sample in a 1.5-mL eppendorf tube, rapidly followed by addition of 0.1 mL of a 30 mM $FeCl_3$ solution in 1.2 M HCl. The tube was closed tightly immediately and vortexed vigorously in the dark for 20 min. Subsequently, the absorption at 650 nm originating from

produced methylene blue (MTB) was measured with UV-vis spectroscopy. A calibration curve was determined with a series of S^{2-} standards with concentrations of 0, 2, 5, 10, 20, 50, and 80 µM prepared from the standardized stock solution of 40.7 mM Na_2S. The presence of Cd^{II} ions did not influence the assay at the concentrations used. (c) *ICP-MS*: ICP-MS was performed with a Varian 820-MS instrument to determine the total sulfur and cadmium concentrations in both, a purified sample of Cd/S-cicMT2 as well as a sample after acidification and degassing. Also Cd_5-cicMT2 was analyzed for comparison.

Spectrophotometric pH titration of Cd/S-cicMT2. 800 µL of a 10 µM sample in 1 mM Tris-HCl (pH 7.5) were titrated with N_2 saturated HCl in volume increments of 0.5-1 µL. After each acid addition, a UV absorption spectrum as well as the pH value of the solution was recorded. The entire titration was carried out under a flow of argon. Origin 7.0 (Origin Lab Corporation, Northhampton, MA, USA) was applied for curve-fitting of the experimental data with pK_a equations as described.[35]

Limited proteolytic digestion of Cd/S-cicMT2 with *Tritirachium album* proteinase K. Limited proteolytic digestion was performed as described previously.[29, 36] The major fraction obtained during SEC-purification of the digestion mixture was further analyzed by amino acid analysis and MALDI-TOF (matrix assisted laser desorption/ionization) mass spectrometry as reported.[29]

RESULTS AND DISCUSSION

Cd^{II} titration of Zn_5-cicMT2 in presence of S^{2-} ions. Zn_5-cicMT2 was overexpressed in form of a fusion protein with a self-cleavable Intein-tag in *E. coli*. In contrast to the results reported with the GST-fusion system, no sulfide ions were detected in the purified protein.[14, 26, 27] Nevertheless, a broadening or shift of the LMCT bands of the Cd^{II}-form at 250 nm towards higher wavelength, i.e. 275 nm, was observed after accidental dialysis of the protein in an untreated dialysis membrane containing residual sulfide ions. The apparent incorporation of sulfide ions into the Cd^{II}-thiolate cluster could be reproduced under defined conditions when Zn_5-cicMT2 was titrated with increasing amounts of Cd^{II} ions in the presence of 10 equivalents of sodium sulfide. An absorption increase of the LMCT bands around 275 nm is observed up to the addition of approximately 9 equiv. of Cd^{II} ions (Figure 1a). In comparison, in absence of sulfide ions only 5 equiv. of Cd^{II} are incorporated.[29] It has to be noted that the shoulder at 275 nm is not observed, when either Zn_5-cicMT2 is mixed with 10 equivalents of Na_2S (dash-dotted line in Figure 1a) or when 10 equivalents of Na_2S are titrated with up to 15 equivalents of Cd^{II}. Interestingly, the apparently incorporation of Cd^{II} ions into cicMT2 takes place although the ionic product $[Cd^{2+}][S^{2-}]$ of the titration solution already by far exceeds the value of the solubility product of CdS, i.e. $1.40 \cdot 10^{-29}$ M^2 at 25 °C,[37] after addition of

the first equiv. of Cd^{II}, i.e. 10^{-9} M^2. To evaluate, if indeed bound sulfide ions are responsible for the increase and shift of the LMCT bands relative to experiments in absence of sulfide, a sample of Cd/S-cicMT2 was acidified to pH 2 with HCl, placed

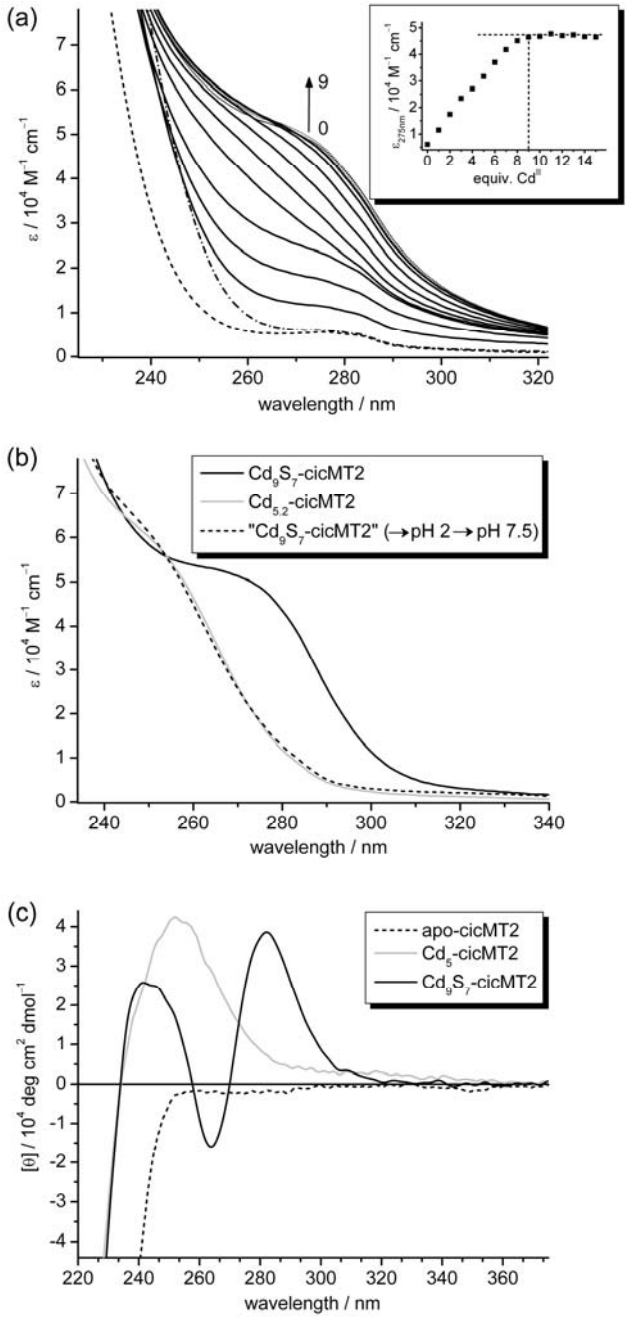

Figure 1. (a) UV spectra of the titration of Zn_5-cicMT2 (dashed line) with increasing equivalents of Cd^{II} in presence of 10 equiv. of S^{2-}. The dash-dotted spectrum is obtained after addition of 10 equiv. of S^{2-} in form of Na_2S to Zn_5-cicMT2. The inset shows the plot of molar absorptivity at 275 nm against the number of Cd^{II} equivalents added. (b) UV spectra of $Cd_{5.2}$-cicMT2 (dashed line), Cd_9S_7-cicMT2 (black), as well as the Cd_9S_7-cicMT2 species after acidification, vacuum centrifugation, and re-neutralisation to pH 7.5 (gray). (c) CD spectra of apo-cicMT2 (dashed line),

$Cd_{5.2}$-cicMT2 (gray), and Cd_9S_7-cicMT2 (black).

in the vacuum concentrator to remove released H_2S, and re-neutralized to pH 7.5. A UV spectrum was taken, which resembles closely the spectrum of Cd_5-cicMT2, indicating that the shoulder at 275 nm was indeed caused by sulfide, and additional Cd^{II}, ions (Figure 1b).

Bathochromic shifts of LMCT bands are characteristic for the formation of cadmium-thiolate clusters in MTs and have been associated with the transition of initially terminal thiolate ligands to bridging ones, i.e. coordination of one thiolate group to two Cd^{II} ions, in the cluster structure.[38] Analogous band shifts to higher wavelengths were also observed for thiol capped CdS particles, when the size of the clusters was increased from approximately 1.8 to 2.5 nm, which is also roughly the size range of MT structures known so far.[39] Broadening of the absorption envelope, observed, e.g., in the sulfide ions containing cluster structure $[Cd_{10}S_4(SPh)_{16}]^{4-}$ to approximately 310 nm, was attributed to LMCT transitions involving the μ_3-S^{2-} ions.[40, 41] *Ab initio* calculations have shown that the HOMO-LUMO differences in cadmium-thiolate clusters decrease with increasing cluster size, but that also the transition to structures with highly coordinated sulfur atoms, e.g. μ_3- or even μ_4-S^{2-} ions, has a major influence.[42, 43]

To further characterize the Cd/S-cicMT2 form a CD spectrum was taken and compared to the sulfide-free Cd^{II}-form (Figure 1c). The CD spectrum of Cd/S-cicMT2 features intense dichroic bands at (+) 244 nm ($[\theta] = 2.5 \cdot 10^4$ deg cm^2 dmol^{-1}), (-) 264 nm ($1.6 \cdot 10^4$ deg cm^2 dmol^{-1}), and (+) 282 nm ($3.9 \cdot 10^4$ deg cm^2 dmol^{-1}) with two inflection points at 255 nm and 272 nm, respectively. The CD profile is thus significantly different from that of Cd_5-cicMT2, which is characterized by a single intense dichroic band at (+) 253 nm ($[\theta] = 4.2 \cdot 10^4$ deg cm^2 dmol^{-1}), corroborating an alteration of the cluster structure in Cd/S-cicMT2 relative to Cd_5-cicMT2. Transitions in the range of 250-300 nm have been also observed in the CD spectra of the recombinantly expressed mammalian MT1 metallothionein upon sulfide incorporation.[22]

For a more detailed characterization, a larger amount of Zn_5-cicMT2 was titrated with Cd^{II} ions in presence of 10 equivalents of sulfide ions and the resulting mixture was subjected to size-exclusion chromatography. The elution profile (Figure 2b) shows clearly that a certain amount of aggregation and dimerization of the protein took place (7.5-9.7 mL), but also a monomeric species is obtained (~10.3 mL). The elution times of Cd_5-cicMT2 and Cd/S-cicMT2 are very similar, suggesting that the two species have similar hydrodynamic radii. To analyse the mass and the size of the two protein forms in more detail, after purification with SEC both were subjected to sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2a). The silver stained gel shows a single band each for Cd_5- and Cd/S-cicMT2 with an apparent molar mass slightly above 27 kDa (lanes 1 and 2). The difference between apparent molar masses and expected masses, i.e. 8.5 kDa for Cd_5-cicMT2, can be explained by only partial denaturation of the protein structure with SDS, while the metal-thiolate clusters are left untouched. This results in lower charge-to-protein mass ratios with respect to the proteins used in the molar mass marker (lanes M).[29, 35] However, the metal clusters can be disrupted by thiol group modification with mBBr.[29, 32, 35] The completely denatured proteins migrate in SDS PAGE at an apparent molecular weight of ~10 kDa (lanes 3 and 4), which is in accordance with the theoretically expected molecular mass of apo-cicMT2 after modification of all 14 cysteine residues (7'969.9 Da + 14*190.2 Da = 10'632.7 Da).[29] Hence, the results from SDS PAGE are in agreement with the results from the chromatogram that both species have the same hydrodynamic radius. In addition, the equal apparent molar masses of the partially SDS denatured proteins (lanes 1 and 2) indicate that the preserved clusters keep the partially denaturated polypeptide chains in similar structural arrangements. I.e. it is unlikely that for example the metal ions in Cd_5-cicMT2 are arranged only in a single cluster, while Cd/S-cicMT2 contains two.

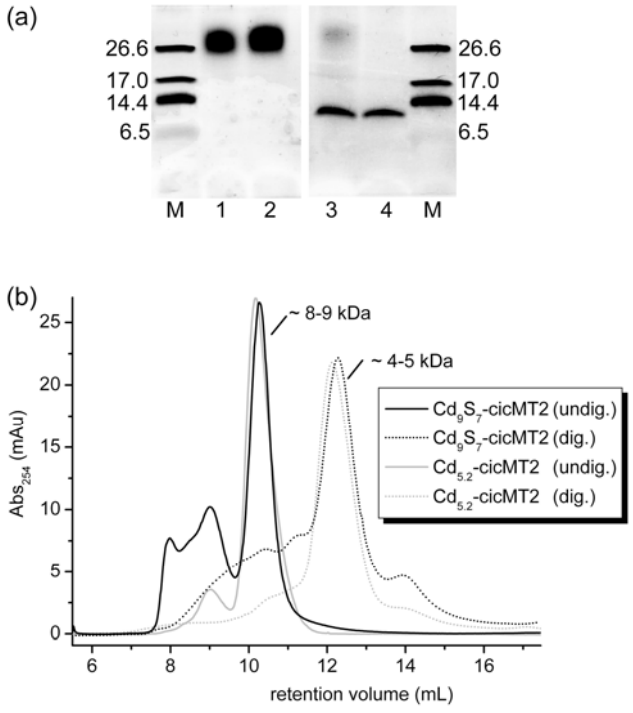

Figure 2. (a) Silver-stained SDS-PAGE (12 %) of unmodified and mBBr-modified $Cd_{5.2}$-cicMT2 (lanes 1 and 4) and Cd_9S_7-cicMT2 (lanes 2 and 3) relative to a peptide marker (lanes marked with M). (b) Size-exclusion chromatogram of undigested and proteinase K digested Cd_9S_7-cicMT2 (black solid and dotted lines) and $Cd_{5.2}$-cicMT2 (gray solid and dotted lines).

Stoichiometry of the Cd/S-cicMT2 complex. To quantify the number of sulfide ions incorporated into Cd/S-cicMT2, the S^{2-} concentration was determined using a combination of three different methods (Table 2). The 2-PDS assay is commonly used in MT research to determine protein concentrations via the quantification of thiolate groups. As 2-PDS undergoes the same reaction with sulfide ions, the assay can be also applied to determine the combined content of Cys thiolate groups and sulfide ions in a protein sample. Cd^{II} ions do not interfere with the assay under the conditions and at the concentrations used in the experiments. Comparison of the combined content of Cys thiolate groups and S^{2-} ions with the protein concentration obtained from amino acid analysis (AAA) yields a sulfide content of approximately 7.4 ions per protein molecule (Tables 2 and 3). To assess the protein concentration directly with the 2-PDS assay, the sulfide ions in Cd/S-cicMT2 were protonated with HCl and subsequently the formed H_2S removed by centrifugation under vacuum. Comparison of the 2-PDS assay results with the values obtained from the same sample

Table 2. Original concentration data for Cd_5-cicMT2 and Cd/S-cicMT2 measured with various methods. All concentrations are given in μM.

	sample	$[Cd^{2+}]$ (F-AAS)	$[Zn^{2+}]$ (F-AAS)	$[-S^-]$ (+ $[S^{2-}]$) (2-PDS)	[cicMT2] (AAA)	$[S^{2-}]$ (MTB)	[total S] (ICP-MS)	$[Cd^{2+}]$ (ICP-MS)
Cd_5-cicMT2	**1**	304(3)	1(1)	822(7)	60.6	---	---	---
	2	---	---	---	---	---	142(1)	44(1)
Cd/S-cicMT2	**1**	164(1)	1(1)	392(1)	18.3	---	---	---
	1[a]	164(1)	1(1)	267(1)	---	---	---	---
	2	79(1)	1(1)	185(1)	---	62(1)	---	---
	3	---	---	---	---	---	111(1)	39(1)
	3[a]	---	---	---	---	---	[33(1)]	[34(1)]

[a]Acidified and vacuum-degassed sample. Errors are given for the 3σ level.

Table 3. Summary of calculated and measured data for the analysis of the Cd^{2+} and S^{2-} content in Cd_5-cicMT2 and Cd/S-cicMT2. All concentrations are given in μM.

	sample	$[Cd^{2+}]$	[cicMT2]	Cd^{2+} : cicMT2	$[S^{2-}]$	S^{2-} : cicMT2	total S : Cd^{2+}
Cd_5-cicMT2	**1**	304(3)	58.7[a], 60.6[b]	5.2, 5.0	---	---	3.5[g], 3.6[g]
	2	44(1)	7.9[c]	5.6	---	---	3.2[g]
Cd/S-cicMT2	**1**	164(1)	18.3[b], 19.1[d]	9.0, 8.6	136[f], 125[f]	7.4, 6.5	2.8[h], 2.9[h]
	2	79(1)	8.8[e]	9.0	62(1)	7.0	2.8[h]
	3	39(1)	---	---	---	---	2.8[i]

[a]Calculated from $[-S^-]$ (+ $[S^{2-}]$) (Table 2, column 5) considering 14 Cys residues per protein

[b]Value from amino acid analysis (Table 2, column 6)

[c]Calculated from [total S] (Table 2, column 8) considering 14 Cys and 4 Met residues per protein

[d]Calculated from $[-S^-]$ (+ $[S^{2-}]$) (Table 2, column 5) of the acidified and vacuum-degassed sample considering 14 Cys residues per protein

[e]Calculated from the difference between ($[-S^-]$ + $[S^{2-}]$) (Table 2, column 5) and $[S^{2-}]$ (Table 2, column 7) considering 14 Cys residues per protein

[f]Calculated from the difference between ($[-S^-]$ + $[S^{2-}]$) (Table 2, column 5) and ($[cicMT2]^{b,d}$ ·14) (Table 3, column 4)

[g][total S] calculated from [cicMT2] (Table 3, column 4) considering 14 Cys and 4 Met residues per protein

[h][total S] calculated from the sum of [cicMT2] (Table 3, column 4) considering 14 Cys and 4 Met residues per protein and [S^{2-}] (Table 3, column 6)

[i]Data taken from Table 2, columns 8 and 9

before acidification yields a S^{2-} content of approximately 6.5 ions per protein molecule. As a third method, the S^{2-} content was determined directly by spectrophotometric measurement of the amount of methylene blue produced upon reaction of the sulfide ions in a strongly acidic solution with *N*, *N*-dimethyl-*p*-phenylenediamine in presence of ferric chloride. In a similar experiment, this method has been also used to determine the sulfide content in the Cd-γ-Glu peptides of *S. pombe*.[44] The colorimetric methylene blue assay yields a S^{2-} content of approximately 7.0 ions per cicMT2 molecule.

The content of Cd^{II} ions was determined with F-AAS and varies between 8.6 and 9.0 per Cd/S-cicMT2 molecule depending on the method used for the determination of the protein concentration (Table 3). The Zn^{II} content was lower than 0.1 equivalents in all samples (Table 2). Coordination of nine Cd^{II} ions per cicMT2 molecule is in agreement with the results from the titration of Zn_5-cicMT2 with Cd^{II} ions in presence of S^{2-} described above (Figure 1a).

Methods that allow the parallel determination of the total sulfur and cadmium concentrations in a sample are ICP-MS and ICP-AES. For the Cd_5-cicMT2 sample the ICP-MS measurement gave a sulfur to Cd^{II} ratio of 3.2 (Table 3). This allows to directly calculate a Cd^{II} content of 5.6 ions per protein molecule as only Cys and Met residues are contributing to the total sulfur content. Hence the Cd^{II} content is higher than values determined with combinations of the 2-PDS assay, amino acid analysis, and F-AAS, although only slightly. For the Cd/S-cicMT2 complex a ratio of 2.8 was determined in agreement with the values obtained with the other methods (Table 3), however, the initially unknown number of S^{2-} ions renders the calculation of the complex stoichiometry impossible without additional methods. A large number of stoichiometries, e.g. $Cd_7S_{1.6}$-, $Cd_8S_{4.4}$-, $Cd_9S_{7.2}$-, $Cd_{10}S_{10}$-cicMT2, or species with even higher Cd^{II} contents are equally consistent with the experimental value. In an effort to determine the sulfide content, also ICP-MS with the same Cd/S-cicMT2 sample after acidification and vacuum-degassing was performed, but the resulting total S content is far too low and would indicate an initial sulfide content far above 30 ions per cicMT2 molecule (Table 2, values given in square brackets). However, the underestimation of sulfur in acidified protein samples with ICP-MS is a known problem reported previously in the literature.[26]

Taken together all results with the different analytical methods (Tables 2 and 3), the composition of the Cd/S-cicMT2 species can be best given as $Cd_{8.9(2)}S_{7.0(5)}$-cicMT2 or approximately Cd_9S_7-

cicMT2.

Effect of sulfide ions on cluster stability. To compare the pH stability of metal ion-binding of Cd_9S_7-cicMT2 with the one of Cd_5-cicMT2, a pH titration was performed and the metal ion release followed with UV spectroscopy by means of the decrease of the LMCT band at 275 nm (Fig. 3). Down to a pH value of ~5.5 the absorption at 275 nm shows only little changes, but decreases rapidly below 5.5. At around pH 1.0 the point of complete Cd^{II} ion release from the protein is reached. The overall apparent pK_a value of the metal-binding ligands, i.e. the Cys thiolate groups

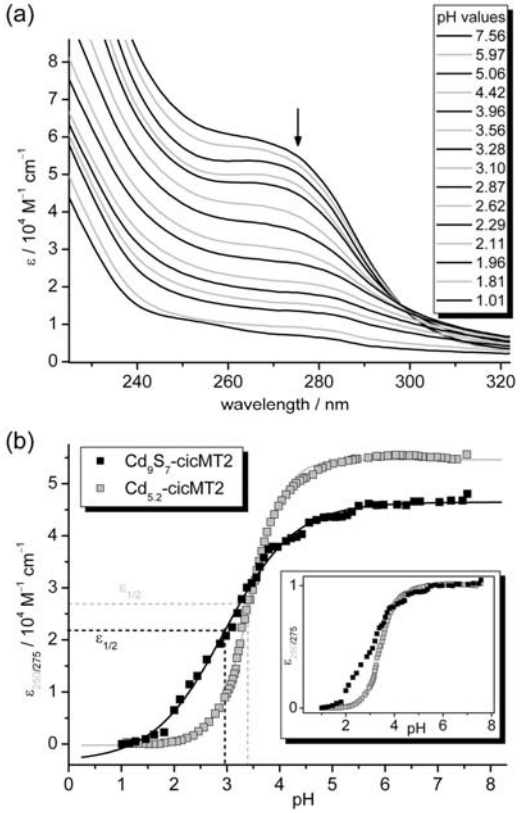

Figure 3. (a) UV spectra of titration of Cd_9S_7-cicMT2 with increasing amounts of HCl. The arrow indicates the decrease in absorptivity at 275 nm. (b) Plots of molar absorptivity at 275 nm or 250 nm against the respective pH value for the pH titration of Cd_9S_7-cicMT2 (black squares) and $Cd_{5.2}$-cicMT2 (gray squares),[29] respectively. Data points are fitted with Eq. (1) (Table 4) to determine the apparent pK_a values of the S-ligands (solid lines). Dotted lines indicate the molar absorptivity value at half maximum absorptivity. The inset shows the plots normalized onto each other to allow for easier comparison.

together with the S^{2-} ions, was determined by curve fitting of the pH titration data as described (Table 4).[35]

Table 4. Apparent pK_a values[a] of Cys residues (and sulfide ions) in Cd_9S_7- and Cd_5-cicMT2

Eq. 1	Cd_9S_7-cicMT2	Cd_5-cicMT2[29]
A_{MT}	46299 ± 265	54607 ± 162
A_{MTH_n}	-3213 ± 749	-225 ± 246
pK	2.95 ± 0.03	3.439 ± 0.006
n	0.66 ± 0.02	1.38 ± 0.03
Eq. 2		
A_{MT}	46662 ± 1675	
A_{MTH_o}	36817 ± 3294	
$A_{MTH_{m+o}}$	15119 ± 2191	
$A_{MTH_{n+m+o}}$	0 ± 1450	
pK_3	4.6 ± 0.5	
pK_2	3.27 ± 0.04	
pK_1	2.09 ± 0.05	
o	0.8 ± 0.3	
m	1.7 ± 0.3	
n	2.1 ± 0.4	

[a]Curve fitting of pH titration data was performed with the following two equations:

$$A_{total} = \frac{A_{MT} + A_{MTH_n} 10^{n(pK-pH)}}{1+10^{n(pK-pH)}} \quad \text{(Eq. 1)}$$

$$A_{total} = \frac{A_{MTH_{m+o}} + A_{MTH_{n+m+o}} 10^{n(pK_1-pH)}}{1+10^{n(pK_1-pH)}} + \frac{A_{MTH_o} + A_{MTH_{m+o}} 10^{m(pK_2-pH)}}{1+10^{m(pK_2-pH)}} \frac{A_{MT} + A_{MTH_o} 10^{o(pK3-pH)}}{1+10^{o(pK_3-pH)}} \quad \text{(Eq. 2)}$$

A_{MT} is the absorptivity of the fully metal ion-loaded protein (=A_{max}), A_{MTH_n} (Eq. 1) or $A_{MTH_{n+m+o}}$ (Eq. 2) denote the value obtained for apo-MT after acidification (=A_{min}), and n, m, and o are measures for the slope of the curves.

With 2.95(3), this value is by nearly 0.5 units lower than the pK_a value obtained for the Cys residues in $Cd_{5.2}$-cicMT2, i.e. 3.439(6).[29] Hence Cd_9S_7-cicMT2 features a higher overall stability against pH-dependent metal loss, obviously brought about by the bound sulfide ions. Similarly, the apparent pK_a values of the Cd-γ-glutamyl peptide complexes from *S. pombe* decreased from ~5.4 to ~3.9 upon incorporation of sulfide ions.[7] Accordingly, the pH stability of both, the sulfide-free and the sulfide-containing Cd^{II} complexes of cicMT2, is significantly higher than for the corresponding

complexes of fission yeast. Taking a closer look at the plots of molar absorptivity against the respective pH value (Fig. 3b) it is apparent that for both species the pH-dependent metal loss starts already below pH values of 5.5. Differences become apparent below approximately pH 3.5 to 3.0, especially when examining the normalized plots (Fig. 3b, inset). While the curve for the pH titration of $Cd_{5.2}$-cicMT2 shows a steep decrease of the absorptivity and a complete metal ion release at pH ~2, the curve for Cd_9S_7-cicMT2 proceeds more shallow and the lowest absorptivity value, i.e. complete loss of Cd^{II} ions, is reached at pH ~1. Accordingly, the metal ion release range is broader for the species containing sulfide ions, and the corresponding decreased steepness of the plot is reflected by the lower value for *n* in Eq. 1 used for the curve fit (Table 4.), i.e. 0.66(2) in Cd_9S_7-cicMT2 compared to 1.38(3) in $Cd_{5.2}$-cicMT2.[35] Having in mind that the free ligands contributing to the LMCT bands at 275 nm have three different pK_a values, i.e. hydrogen sulfide 7.02 and 14.9 and the Cys thiol groups 8.57, we also attempted a curve fit of the pH titration data with an equation considering three different pK_a values (Eq. 2, Table 4). Disregarding the highest pK_a value of 4.6(5), which is on one hand relatively poor defined and on the other hand also observed in experiments with MTs devoid of sulfide ions, we can deduce two well-defined pK_a values. The pK_a value of 3.27(4) is similar to the apparent pK_a value of the Cys residues in $Cd_{5.2}$-cicMT2 (3.439(6)) and hence can be assigned to the Cys thiolate groups in Cd_9S_7-cicMT2. The pK_a value of 2.09(5) should then arise from the incorporated sulfide ions. Assuming that the pK_a values of both ligands, Cys thiolate groups and sulfide ions, show a decrease in presence of Cd^{II} ions of similar magnitude, i.e. by roughly 5 pK_a units, the value of 2.09(5) could indeed originate from the second pK_a value of hydrogen sulfide (7.02). This view is corroborated by the respective absorptivity decrease accompanying the titration steps. The absorption decrease caused by the protonation of the 14 Cys thiolate groups amounts to ~31'500 M^{-1} cm^{-1} (= 46'662 - 15'119), while the absorption decreases during the final protonation of the seven coordinated sulfide ions by ~15'100 M^{-1} cm^{-1} and thus roughly by 50% of the value observed for the Cys residues in agreement with the number of protonated ligands in both cases.

Cluster arrangement in Cd_9S_7-cicMT2. The metal-thiolate clusters of metallothioneins have been shown to protect the peptide backbone to a certain extent against proteolytic cleavage.[29, 36, 45, 46] In addition, the metal-thiolate clusters usually stay intact at physiological pH even if backbone cleavage occurs between amino acid residues belonging to the same cluster. Digestion of the sulfide-free $Cd_{5.2}$-cicMT2 form with the subtilisin–related serine protease *T. album* proteinase K already showed that the N- and C-terminal Cys-rich regions of the protein are co-eluting from the

size exclusion chromatography column corroborating the view that the Cd^{II} ions are coordinated in a single metal-thiolate cluster arrangement.[29] In order to evaluate the number and size of metal clusters formed in Cd_9S_7-cicMT2, an analogous digestion experiment was performed. The subsequent separation of the digestion mixture with gel filtration under non-denaturing conditions results in a broad elution profile with a major sharp peak at 12.5 mL (Fig. 2b). The elution volume of this peak is significantly larger than for the undigested protein (10.3 mL), but is nearly identical to the one observed for the digested $Cd_{5.2}$-cicMT2 species. The ratio between S from Cys thiolate groups and S^{2-} ions and Cd^{II} ions was determined in the peak fraction with the 2-PDS assay and F-AAS, respectively, and yields a value of 2.3, which is the same as for the undigested protein. Hence it can be assumed that neither sulfide ions, nor Cys residues, or Cd^{II} ions were lost from the cluster during the digestion procedure. The MALDI-TOF spectrum collected of the major peak fractions exhibits three major signals, one at 2004.7 Da matching the mass for residues 62-81 (calc. 2005.2 Da), one at 2133.7 Da corresponding to residues 61-81 (calc. 2133.8 Da) and the third one at 2523.7 Da corresponding to residues 2-28 (calc. 2523.8 Da, Fig. 4). The assignment was further confirmed by amino acid analysis (Suppl. Mat.). Quantification of Cys residues was not possible. Hence all available data strongly suggests the presence of a single metal cluster in Cd_9S_7-cicMT2 in analogy to $Cd_{5.2}$-cicMT2. A metal cluster with comparable stoichiometry is realized in the model compound $[S_4Cd_{10}(SPh)_{16}]^{4-}$ (Fig. 4c).[47] The structure analysis of $[S_4Cd_{10}(SPh)_{16}]^{4-}$ shows that the ten Cd^{II} ions and 20 sulfur ligands constitute four fused adamantanoid cages with six inner Cd^{II} ions capped by the four sulfide ligands and the other four Cd^{II} ions by 16 thiolate ligands. Considering the similarity between Cd_9S_7-cicMT2 and $[S_4Cd_{10}(SPh)_{16}]^{4-}$ with respect to the total amount of coordinated metal ions and sulfur ligands, i.e. 21 in Cd_9S_7-cicMT2 compared to 20 in $[S_4Cd_{10}(SPh)_{16}]^{4-}$, the formation of just a single metal cluster in Cd_9S_7-cicMT2 is theoretically feasible. In addition, the Cys-free linker region in cicMT2 connecting the N- and C-terminal Cys-rich regions is long enough to easily enable the accommodation of such a larger cluster without a major change in overall protein size. It should be emphasized however, that the detailed structures of both clusters in the model compound and in cicMT2 will significantly differ from each other especially considering the different amounts of sulfide ions and thiolate ligands in both compounds. A second aspect to consider is the relatively large negative charge of the proposed Cd_9S_7-cicMT2 structure of -12 assuming the presence of exclusively S^{2-} ions, which would require some sort of charge compensation. The overall charge would be significantly smaller supposing protonation of the sulfide ions to hydrogen sulfide, HS^-. This assumption would be in-line with the observation during the pH titration of the complex (Fig. 3 and Table 4), which shows that the first protonation

step assigned to the protonation of the Cys residues leads to a 2/3 absorptivity decrease while the second protonation step might correspond to protonation of the sulfide ions or possibly HS^-. Accordingly it is tempting to say that while the protonation of the Cys residues requires 14 protons, the protonation of the (hydrogen) sulfide ions requires only 50% of this value, i.e. seven protons in line with seven HS^- ions. However, the exact charge and hence the protonation states of the different ligands needs to be established.

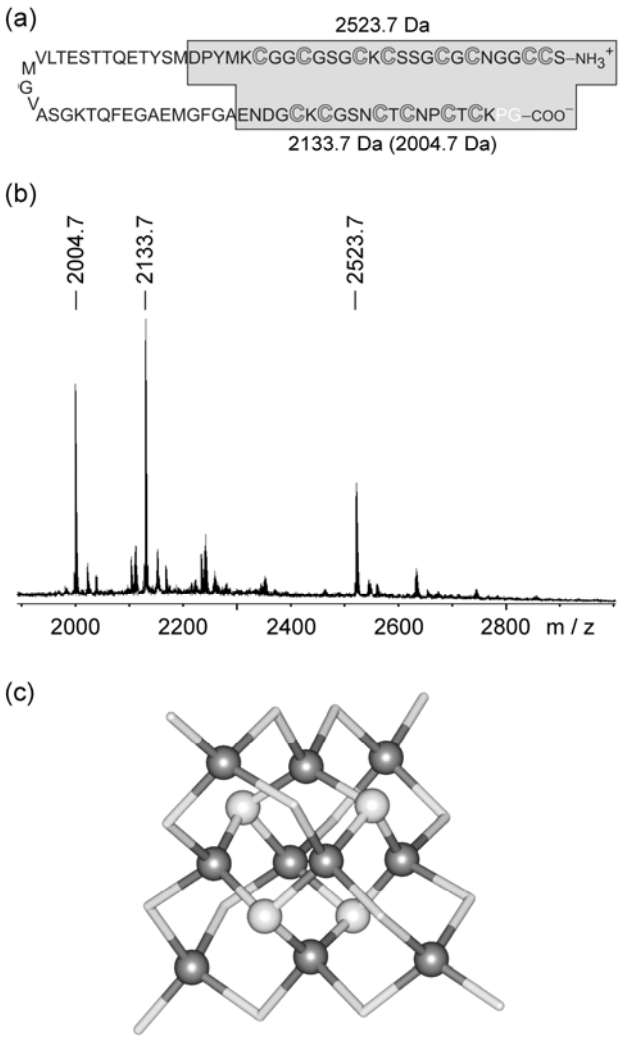

Figure 4. (a) Amino acid sequence of cicMT2 with the Cys residues highlighted. The vector-derived C-terminal ProGly residues are indicated with white letters. The two Cys-rich parts of the protein coordinating the Cd^{II} ions after proteolytic digestion with proteinase K are enclosed by a gray box and the respective molecular weight of the fragments is given. 2004.7 kDa is the weight of the C-terminal fragment after additional cleavage or the N-terminal Glu. (b) MALDI-TOF analysis of the peak fraction at 12.5 mL elution volume (Fig. 2b) of the proteinase K digested Cd_9S_7-cicMT2 sample. (c) Core structure of $[S_4Cd_{10}(SPh)_{16}]^{4-}$.[47] Cd^{II} ions are depicted as dark-gray spheres, S^{2-}

ions as light-gray spheres and the thiolate groups of the ligands as sticks. Phenyl rings are omitted for clarity.

CONCLUSIONS

While phytochelatins are known as efficient Cd^{II}-chelators in plants,[48] the results presented here provide evidence that also the Cd^{II} binding capacity of plant MTs such as cicMT2 can be enhanced in the presence of sulfide ions. The ratio of S-ligands, i.e. Cys thiolates and sulfide ions, to coordinated Cd^{II} ions in the here described Cd_9S_7-cicMT2 species is 2.3 and hence lower than ratios usually observed for MTs from various species, e.g. 2.9 for mammalian MTs or 2.8 for the sulfide-free Cd_5-cicMT2 form. For the sulfide containing complexes of the fission yeast *S. pombe*, i.e. $Cd_{5.4}S[(\gamma\text{-EC})_3G]_4$ and the *in vitro* reconstituted complex, as well as for the sulfide containing high molecular weight complex from the plant *R. serpentina* ratios of 2.4, 2.2 and 1.9, respectively, are observed. [4, 5, 13] Hence the here proposed stoichiometry is well in line with the composition of other Cd/S complexes previously described. In addition to the observation that the incorporation of sulfide ions increases the Cd^{II} binding capacity of the plant MT cic-MT2, it also leads to significantly higher pH stability, and hence recruitment of sulfide ligands might indeed be a relatively inexpensive way for the cell to enhance the effectiveness of Cd^{II} detoxification. Further experiments will have to show, if the Cd^{II} binding capacity of cic-MT2 can be even further increased if higher initial sulfide concentrations are provided.

ASSOCIATED CONTENT

Supporting Information

Results of AAA for the proteinase K digested Cd_9S_7-cicMT2 species. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table of Contents Synopsis

The Cd^{II} binding capacity of the plant metallothionein2 from *Cicer arietinum* (chickpea), cic-MT2, can be nearly doubled upon incorporation of sulfide ions into the metal-thiolate cluster. The resulting cluster arrangement featuring the stoichiometry $Cd_9S_7Cys_{14}$ shows distinctively different features in the circular dichroism spectra and a significantly increased pH stability compared to the sulfide-free form. Sulfide incorporation into the metal-thiolate clusters might be an economic and efficient strategy to increase the detoxification capacity of metallothionein.